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The Measurement of Triphosphopyridine Nucleotide and Reduced Triphosphopyridine Nucleotide and the Role of Hemoglobin in Producing Erroneous Triphosphopyridine Nucleotide Values*

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SUMMARY

A method is described for determining TPN$^+$ and TPNH in a single tissue homogenate made in dilute NaOH at 0°. One portion, for TPN$^+$, is acidified after adding ascorbic acid to prevent TPNH oxidation. A second portion, for TPNH, is heated (still alkaline) after adding cysteine to prevent oxidation. If desired, a third portion can be analyzed directly without heating for total TPN (TPN$^+$ plus TPNH). The same homogenate can also be used for measuring DPN$^+$ and DPNH. In all cases the actual analyses are made with enzymatic cycling. Consequently, by reason of the high sensitivity, it is unnecessary to deproteinize. The procedure presented avoids the danger of TPNH oxidation to TPN$^+$ by tissue hemoglobin which was responsible for our earlier erroneous conclusion that there exists an acid-labile tissue form of TPN$^+$.

The oxidation of TPNH by hemoglobin has been studied intensively. The oxidation by free hemoglobin occurs to the same degree in strong acids as in dilute. Therefore, the possibility of obtaining erroneously high TPN$^+$ values is not eliminated by the preparation of homogenates in strong acid, although TPN$^+$ formation is greatly decreased if red blood cells in the tissue are intact at the time of strong acid addition. Neither is the danger eliminated by preparation of extracts with heat at neutral pH.

With the use of the revised procedure the levels of TPN$^+$ and TPNH were measured in liver, kidney, heart, brain, and blood. The ratio of TPNH to TPN$^+$ was found to vary from 20 to 1 in heart to 3 to 1 in blood.

There is an increasing interest in the true levels *in vivo* of biologically labile metabolites and cofactors, and in the changes that may occur in different physiological and pathological states. The measurement of pyridine nucleotides in tissues has caused special difficulty owing to the problem of preparing material for analysis without losses and without uncertainty as to whether oxidation or reduction may have occurred. The difficulties arise primarily because DPNH and TPNH are unstable in acid, whereas DPN$^+$ and TPN$^+$ are unstable in alkali. Consequently, it has been the usual practice to prepare two extracts, one in acid for the oxidized forms and one in alkali for the reduced forms. Alternatively, a single neutral extract prepared with heat has been used (1, 2). More recently Neuhoff (3) has found it possible to prepare a neutral extract without heat, which contains both oxidized and reduced forms, by homogenizing tissues in phenol-saturated buffer; and Heldt, Klingenberg, and Papenberg (4) have utilized a single acid extract in which the degradation products of DPNH and TPNH are measured after chromatographic separation. In all cases there is a real danger of oxidation of DPNH or TPNH at some point in the process. Thus, experiments to be presented appear to indicate some TPNH oxidation when liver is heated in neutral solution, and it was found earlier (5) that blood can oxidize TPNH in alkaline solution when heated to destroy tissue enzymes. Finally, the present report concerns the special danger of oxidation of TPNH during the preparation of acid homogenates.

We have reported that much more TPN$^+$ is found in tissue homogenates prepared in dilute acid than in those prepared with a strong acid protein precipitant (6). This led to the conclusion that there existed in tissues an acid-labile form of TPN$^+$. We now believe, in agreement with Neubert, Schulz, and Hoehne (7) and Heldt *et al.* (4, 8), that this conclusion was erroneous. It appears from present results that hemoglobin, upon acidification, can oxidize TPNH or DPNH (to TPN$^+$ or DPN$^+$) faster than the acid can destroy them.

Moreover, the oxidation is nearly stoichiometric and occurs even at extreme hemoglobin dilution. The oxidation can occur in strong or weak acid, but if the hemoglobin is present in intact red blood cells, a strong acid protein precipitant can destroy hemoglobin with only slight oxidation of the reduced nucleotides. This is presumably due to prevention of contact between hemo-

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globin and the nucleotides until they have been decomposed by the acid.

It has been found possible to avoid the danger of oxidation by hemoglobin and at the same time provide a simplified procedure for measuring tissue pyridine nucleotides.

MATERIALS AND EQUIPMENT

Standard Solutions—TPNH stock solutions are prepared at approximately 5 mM concentration in 0.08 M Na_2CO_3-0.02 M $NaHCO_3$ (pH 10.6) and heated 10 min at 60° to destroy any TPN^+ present. These are standardized by diluting 50-fold in 0.1 M Tris-HCl, pH 8.0, containing 1 mM α-ketoglutarate and 0.03 mM ammonium acetate. After measuring the optical density at 340 mμ, a small volume of solution containing glutamic dehydrogenase is added to give an enzyme concentration of 20 μg per ml. A second reading is made after the TPNH has been oxidized (5 min or less). The stock standard is stored at 4° or below −50° but not at an intermediate temperature (4). After storage for more than a few days at 4° it may be necessary to reheat to destroy TPN^+ that may have been formed and to restandardize. Working standards are diluted daily in 0.02 N NaOH containing 0.5 mM cysteine·HCl (freshly added) and these are kept on ice until used. TPN^+ stock solutions are prepared in H_2O at 5 mM or greater concentration. (For stability, the pH should be in the 4 to 6 range.) These are standardized by diluting to about 0.1 mM concentration in Tris buffer, pH 8, containing 1 mM glucose-6-P. After measuring the optical density at 340 mμ, a small volume of solution containing glucose-6-P dehydrogenase is added to give a concentration in the order of 1 μg per ml. A second reading is made after complete reduction of the TPN^+ (less than 1 min). Stock TPN^+ solutions are ordinarily stable indefinitely if frozen at −20° or below. Solutions more dilute than 1 mM are prepared daily.

Cycling Reagent—The stock cycling reagent (9) is 0.1 M Tris-HCl buffer, pH 8.0, containing 5 mM α-ketoglutarate, 1 mM glucose-6-P, 0.1 mM ADP, 25 mM ammonium acetate, and 0.2 mg of bovine plasma albumin per ml. The stock reagent is conveniently stored at −50° or below in aliquots sufficient for 1 day of use. (At −20° α-ketoglutarate is not indefinitely stable in the reagent.) Before use, glutamic dehydrogenase and glucose-6-P dehydrogenase are added to give respective concentrations of 100 μg per ml and 50 μg per ml. (The latter refers to currently available preparations of about 30% purity.[1]) Since sulfate is inhibitory it is convenient to use glutamic dehydrogenase prepared in glycerin (available from Boehringer and Sons). In the case of glucose-6-P dehydrogenase, the enzyme suspension is centrifuged, as much of the supernatant $(NH_4)_2SO_4$ solution as possible is removed, and the enzyme is dissolved in 2 M ammonium acetate.

Other Reagents—Strong ascorbic acid and cysteine-HCl solutions are indefinitely stable in the frozen state. The 6-P-gluconate dehydrogenase reagent is 0.02 M Tris-HCl buffer, pH 8.0, containing 0.03 M ammonium acetate, 0.1 mM EDTA, 0.03 mM TPN^+, 0.02% bovine plasma albumin, and 1 μg per ml of 6-P-gluconate dehydrogenase (Boehringer and Sons). This should oxidize 6-P-gluconate, if added to a concentration of 0.005 mM, with a half-time of 3 to 6 min.

Fluorometry—The method is described for the Farrand photofluorometer model A which is a filter instrument with 1-ml samples in 3-ml Pyrex test tubes (10 × 75 mm). Precautions and changes that can be made to provide increased stability and reproducibility of reading and minimal optical blanks have been described (10). With a tungsten light source the primary filter is Corning No. 5840; the secondary is No. 3387 plus No. 4303. With a mercury arc light the primary is No. 5860 and the secondary is No. 3387 plus Nos. 4308 and 5562.

ANALYTICAL PROCEDURE

Principle—TPNH is very stable in alkali and TPN^+ is stable in weak alkali for a short time at 0° (see below). Therefore, the same homogenate can be used for the measurement of both TPN^+ and TPNH. TPNH is measured in the alkaline homogenate after heating to destroy TPN^+. Conversely TPN^+ is measured after destroying TPNH with acid. To prevent oxidation of TPNH by hemoglobin during acidification, ascorbic acid is added to the sample beforehand. Similarly oxidation during heating in alkali is prevented by cysteine added to the alkali used for making the homogenate (5). The actual analysis is made by enzymatic cycling (9) which provides sufficient sensitivity and specificity to make deproteinization unnecessary. In the cycling process TPN is alternatively oxidized and reduced in rapid succession with the aid of glutamic dehydrogenase and glucose-6-P dehydrogenase, respectively. After 10,000 cycles, for example, one of the products, 6-P-gluconate, is measured in a separate step with the help of 6-P-gluconate dehydrogenase and an excess of TPN^+. The over-all result is the formation of 10,000 molecules of TPNH for every molecule of TPN^+ or TPNH originally present.

Since the cycling reaction does not distinguish TPN^+ from TPNH, total TPN (*i.e.* TPN^+ and TPNH together) can also be measured directly by analyzing unheated alkaline homogenate. Although the cold weak alkali does not destroy all potentially disturbing enzymes, these are rendered impotent in most tissues by the extreme dilution during cycling.

Analysis of Liver

Preparation of Material—A 50-mg sample of quick frozen liver is homogenized rapidly in 5 ml of 0.04 N NaOH containing 0.5 mM cysteine (NaOH-cysteine) at 0°. (*a*) For *total TPN* (TPN^+ and TPNH together) 200 μl of the homogenate are diluted at 0° with 4 ml of NaOH-cysteine. Care is taken to keep samples at 0°, and the cycling step is started if possible within 30 min. (*b*) For *TPNH* a portion of the diluted homogenate from (*a*) is heated 10 min at 60°. (*c*) For *TPN^+* 5 μl of 1.2 M ascorbic acid are added at 0° to 200 μl of the original homogenate, to give an ascorbic acid concentration of 30 mM. After mixing, the sample is acidified with 2 ml of 0.02 N H_2SO_4-0.1 M Na_2SO_4 (acid-sulfate) and is heated 30 min at 60°. Samples are acidified within 30 min, and prior to this time they are kept on ice.

Appropriate standards would ordinarily be 20 and 40 μl of 0.1 mM TPN^+, or 20 and 40 μl of 1 mM TPNH added at 0° to 5-ml volumes of NaOH-cysteine. The additions are made at the same time that the homogenates are prepared. Thereafter, aliquots of these standards, together with blank aliquots of NaOH-cysteine, are carried through exactly the same procedure

[1] Certain lots of yeast glucose-6-P dehydrogenase, all from Boehringer and Sons, have been found to contain a contaminant which appears to bind a small amount of TPN^+ or TPNH and which decreases the cycling rate at lowest TPN levels. In this case the yield of product is not proportional to TPN concentration, being disproportionately greater at higher TPN levels. (Personal communication from Dr. Janet V. Passonneau.)

TABLE I

Oxidation of TPNH to TPN+ by addition of blood to homogenates of liver

Samples of frozen liver (100 mg) were homogenized at 0° in 20 ml of acid-sulfate (0.02 N H_2SO_4-0.1 M Na_2SO_4) or NaOH-cysteine (0.04 N NaOH-0.5 mM cysteine). Hemolyzed blood or TPNH or both were added, as shown. The blood was added 2 min after homogenizing; the TPNH was added at 3 min. At 4 min, all samples were diluted 10-fold in acid-sulfate, heated immediately for 30 min at 60°, and analyzed for TPN+ by enzymatic cycling (see analytical procedure).

Homogenate	Blood added	TPN+ found		Conversion of added TPNH to TPN
		In liver homogenate	In liver homogenate plus TPNH[a]	
	μl/g liver	*10^{-9} moles/l*[b]	*10^{-9} moles/l*[b]	%
Acid-sulfate	0	128	144	5
Acid-sulfate	7	140	163	7
Acid-sulfate	23	156	200	13
Acid-sulfate	110	172	303	38
NaOH-cysteine	0	133	200	20
NaOH-cysteine	7	152	210	17
NaOH-cysteine	22	173	250	22
NaOH-cysteine	108	214	370	46

[a] The TPNH added was equivalent to 343 × 10^{-9} M concentration at the time of heating in acid.

[b] Expressed as concentration at the time of heating in acid.

as the samples. (TPNH standards are appropriate in the case of analyses for total TPN as well as for TPNH.)

Cycling Steps—Fluorometer tubes containing 100 μl of cycling reagent are placed in a rack in an ice bath. To each tube are added 2 μl of sample, standard or blank in the case of total TPN or TPNH analyses, and 4 μl of each in the case of TPN+ analyses. The rack of tubes is incubated in a water bath at 38° for 1 hour, which is long enough to provide at least 10,000-fold amplification by the cycling process. The reaction is terminated by placing the rack of tubes for 2 min in a boiling H_2O bath. After cooling, 1 ml of 6-P-gluconate dehydrogenase reagent is added, and the final fluorometric reading is made as soon as the dehydrogenase reaction is complete (20 to 60 min). Some preparations of the dehydrogenase have been found to be contaminated with glutamic dehydrogenase which results in a creeping blank. In this case the time between addition of dehydrogenase reagent and reading has to be kept nearly the same for each tube.

The over-all tissue dilution during the cycling step is about 100,000-fold for the TPNH and total TPN samples and 25,000-fold for the TPN+ samples. This results, in the case of normal liver, in TPNH or total TPN concentrations of about 5 × 10^{-9} M and TPN+ concentrations of about 2 × 10^{-9} M. With amplification by cycling of 10,000-fold, and final dilution of another 10-fold, the final TPNH concentrations in the fluorometer tube will be in the range of 2 × 10^{-6} M to 5 × 10^{-6} M. It is good practice to add a 6-P-gluconate standard at the final step, within this range of concentration, as a check on the adequacy of gluconate dehydrogenase and the cycling efficiency.

Analyses of Other Tissues

Kidney, heart, brain, and blood have been analyzed by the method described for liver with apparently satisfactory results (Table X). The only change that seems necessary is to decrease the over-all dilution and adjust concentrations of standards according to anticipated TPN+ and TPNH levels. For *kidney* and *heart*, which have about 0.25 as much total TPN as liver, it is convenient to homogenize the tissue at a dilution of 1:50 (instead of 1:100) and to increase the size of aliquots used for cycling to 4 μl. *Brain*, which contains less than 0.1 as much total TPN as liver, can be homogenized at a dilution of 1:20, and 4-μl aliquots are used for cycling. Aliquots larger than 4 μl can be used, if necessary, but the increased sulfate concentration will decrease the cycling rate (9). If, however, standards are carried through all steps, as recommended, the sulfate will affect standards and samples alike. *Blood* can be diluted initially with 20 volumes of NaOH-cysteine. The subsequent dilutions in acid-sulfate or NaOH-cysteine can both be 1:10, with 4-μl aliquots used for cycling. If blood is diluted less than 1 to 200 at the time of heating in alkali, some loss of TPNH may occur in spite of the presence of cysteine (5).

EXPERIMENTAL PROCEDURE

Oxidation of TPNH by Blood upon Acidification—When frozen rat liver is homogenized in dilute acid at 0° and heated to destroy TPNH a substantial amount of TPN+ is found on analysis (Table I).[2] If hemolyzed blood is added to the cold acid homogenate before all the TPNH of the liver has been destroyed there is a marked increase in the amount of TPN+ found. If TPNH is added to the acid liver homogenate, it also is partially oxidized, especially if extra blood is added (Table I). Oxidation of TPNH to TPN+ is even greater if homogenates are made in dilute alkali (NaOH-cysteine) with subsequent acidification.

These results suggested that the TPN+ levels found in dilute acid homogenates of frozen liver are higher than true values *in vivo* as the result of TPNH oxidation by blood inevitably present. Homogenates of liver made with strong acid contain less TPN+ than those made with dilute acid (6). Therefore, if the above suggestion is correct, blood should oxidize less TPNH when blood-TPNH mixtures are acidified with concentrated acid than with dilute. Experiments made at 0° with hemolyzed blood showed, however, that this is not true (Table II). As much TPNH is oxidized to TPN+ in 0.3 N $HClO_4$ or HCl as in dilute H_2SO_4 at pH 2.3. Even in 1 N $HClO_4$, or 1 N HCl, TPN+ formation was only diminished slightly (not shown). An entirely different result occurs with non-hemolyzed blood. Oxidation to TPN+ was found to be greatest in dilute HCl and fell off to 4% in 0.3 N $HClO_4$. Similar results were obtained by Neubert *et al.* (7). This suggests that stronger acids destroy the TPNH or the offending substance or both in the intact red cells (presumably hemoglobin) before they have a chance to react. $HClO_4$, in addition, would precipitate the hemoglobin in the red cells and further delay contact with TPNH. It will be seen that acidification with dilute H_2SO_4 resulted in very little TPNH oxidation to TPN+ with intact red cells (Table II). This is attributed to the 0.1 M Na_2SO_4 present which renders the solution almost isotonic, thereby delaying hemolysis until most of the TPNH has been destroyed.

Nature of TPNH Reaction with Blood and Hemoglobin—It was

[2] The weak acid used was 0.02 N H_2SO_4 containing 0.1 M Na_2SO_4 which raises the pH to about 2.3. This particular combination is stressed throughout this paper since by its use it is possible to destroy DPNase (TPNase) with heat without appreciable destruction of DPN+ or TPN+ (5).

thought possible that the danger of oxidation of TPNH to TPN^+ by blood might be minimized by preparing homogenates at very high dilution. Therefore experiments were made in which both blood and TPNH concentrations were varied over a wide range (Table III). Two things became apparent. (*a*) Extreme dilution, beyond any practical situation, did not diminish the oxidation, and (*b*) the degree of oxidation to TPN^+ was governed by the ratio of blood to TPNH. The TPN^+ formed approached 8 mmoles per liter of blood with highest ratios of TPNH to blood. Since blood contains 8 to 10 mmoles of hemoglobin (as iron) per liter, the results suggested that blood hemoglobin is responsible for the TPNH oxidation and that the reaction is stoichiometric. An experiment with crystalline hemoglobin (Table IV) showed in fact that hemoglobin is fully capable of oxidizing TPNH to TPN^+ upon acidification and that the oxidation approaches a mole for mole relationship when TPNH is in excess. This was confirmed in numerous other experiments (not shown).

Hemoglobin does not retain in acid its ability to oxidize TPNH to TPN^+. At pH 2.3 this ability is lost with a half-time of about 15 sec (Table V).

Rate of Reaction between Hemoglobin and TPNH—The kinetic situation during the reaction between TPNH and hemoglobin is complicated by the fact that both reactants are unstable in acid. It has just been seen, for example, that the reactivity of hemoglobin is almost gone by the end of 1 min at pH 2.3 and 0°. TPNH under identical conditions (in the absence of hemoglobin) was found to be destroyed (and therefore no longer convertible to TPN^+) with a half-time of 7 min. In spite of these complications a rough estimate of the rate of reaction between hemoglobin and TPNH was made by capitalizing on the fact that ascorbic acid can stop the reaction (see below).

An alkaline solution containing a 0.2 mM concentration each of TPNH and hemoglobin (calculated as iron) was diluted 10-fold at 0° with 0.02 N H_2SO_4-0.1 M Na_2SO_4 (pH 2.3) in each of a number of tubes. At short intervals, beginning at 4 sec, ascorbic acid was added to a concentration of 3 mM to stop the reaction. Any TPNH remaining was destroyed by heating. The results showed the initial velocity of TPN^+ formation to be about 0.03 mmole per liter per min. If the reaction is second order this would give an estimate for the rate constant of 8×10^4 liter moles^{-1} min^{-1}. Even this very large constant would not explain the large fraction of TPNH oxidized to TPN^+ at extreme dilutions of hemoglobin and TPNH. For example, with 0.01 ml of blood per liter (0.1 μM iron) and 0.35 μM TPNH (Table III) it can be calculated that the reactivity of hemoglobin would be lost

TABLE II

Oxidation of TPNH by acidified blood

TPNH was added at 0° to rat blood that had been diluted 50-fold in 0.01 M phosphate buffer, pH 7.2 (Hemolyzed), or isotonic NaCl buffered with 0.01 M phosphate (Non-hemolyzed). After 2 min samples were further diluted 10-fold in the various acids at the temperatures shown and held for 5 min. The 0° samples were brought to room temperature for 10 min to make sure that no TPNH remained. The initial TPNH concentration was 28 μM at this dilution. The TPN^+ formed was measured directly in the fluorometer by adding 100-μl aliquots to 1 ml of 0.1 M Tris-HCl buffer, pH 8.3, containing 1 mM glucose-6-P, and 0.7 μg of glucose-6-P dehydrogenase.

Acid	Temperature	Conversion of added TPNH to TPN^+	
		Hemolyzed	Non-hemolyzed
		%	%
0.02 N H_2SO_4[a]	0°	55	2.6
0.02 N H_2SO_4[a]	25	56	
0.02 N H_2SO_4[a]	60	41	
0.04 N HCl	0	80	40.0
0.3 N HCl	0	69	18.0
0.3 N HCl	25	70	
0.3 N HCl	60	64	
0.3 N $HClO_4$	0	58	3.9

[a] Also containing 0.1 M Na_2SO_4.

TABLE III

Oxidation of TPNH to TPN^+ in acid by various dilutions of blood

Different amounts of hemolyzed rat blood were added at 0° to 0.04 N NaOH containing the concentrations of TPNH indicated below. Aliquots were added at 0° to 5 volumes of the acids shown, and heated at 60° for 30 min. Analyses for the TPN^+ formed were made by direct fluorometric measurement, (see Table II) or, for the lowest TPNH concentration, by enzymatic cycling. Blood dilutions and pyridine nucleotide concentrations shown are those at the acid step.

Acid	Blood dilution	TPNH added		TPN^+ formed	
	ml/l	*μM*	*mmole/l blood*	*μM*	*mmole/l blood*
0.02 N H_2SO_4[a]	0.26	40	157	2.3	8.8
0.02 N H_2SO_4[a]	0.48	40	82	3.3	6.9
0.02 N H_2SO_4[a]	1.0	40	40	6.4	6.4
0.02 N H_2SO_4[a]	0.25	22	88	2.0	8.0
0.02 N H_2SO_4[a]	0.25	38	152	2.2	8.8
0.02 N H_2SO_4[a]	0.21	67	318	2.3	10.9
0.1 N HCl	0.21	22	105	1.3	6.1
0.1 N HCl	0.42	22	53	2.1	4.9
0.1 N HCl	0.84	22	26	3.8	4.5
0.02 N H_2SO_4[a]	0.0025	0.35	140	0.016	6.6
0.02 N H_2SO_4[a]	0.011	0.35	32	0.051	4.7
0.02 N H_2SO_4[a]	0.041	0.35	9	0.19	4.7

[a] Containing also 0.1 M Na_2SO_4.

TABLE IV

Oxidation of TPNH to TPN^+ by hemoglobin in acid solution

Crystalline hemoglobin was prepared from hemolyzed rat blood and analyzed in 0.2% NH_4OH solution by its absorption at 540 mμ. The hemoglobin was added at three concentrations to 0.02 N NaOH containing TPNH; 200 μl of each mixture were added to 1 ml of 0.02 N H_2SO_4-0.1 M Na_2SO_4 and heated 20 min at 60°. TPN^+ resulting from oxidation of TPNH was measured by direct fluorometry (see Table II). The concentrations are recorded for the dilution obtaining after acidification. Hemoglobin is calculated as micromoles of iron per liter.

Hemoglobin	TPNH added	TPN^+ formed	Ratio of TPN^+ to hemoglobin
μM	*μM*	*μM*	
6.3	21.4	5.9	0.94
12.5	21.4	9.9	0.80
25.5	21.4	13.8	0.55

TABLE V

TPNH-oxidizing capacity of hemoglobin and and its loss in acid

Rat hemoglobin was added to 0.02 N H_2SO_4-0.1 M Na_2SO_4 at 0° to give a concentration of 17 μM (as iron). TPNH was added after the time interval shown at an initial concentration of 32 μM. After the last addition, all samples were heated for 15 min at 60° and the TPN^+ was measured as for Table II.

Time of TPNH addition	TPN^+ formed from TPNH
min	%
0.03	29.4
0.25	12.8
0.5	10.2
1.0	4.2
2.0	2.8
3.0	2.9
5.0	2.1

TABLE VI

Protection of TPNH from oxidation by hemoglobin at different pH values

To 130 μM TPNH in 0.04 N NaOH at 0° was added rat hemoglobin at a concentration of 1.2 mg per ml (75 μM iron), with or without ascorbic acid. After 30 sec (except as noted) aliquots of 200 μl were added to 1 ml of either 0.1 M acetate buffer, pH 4.6, 0.02 N H_2SO_4-0.1 M Na_2SO_4 (pH 2.3), or 0.1 N HCl (pH 1.1). These were heated for 20 min at 60° and were analyzed for TPN^+ as in Table II. The ascorbic acid concentrations are those after acidification.

Ascorbic acid	TPN^+ formed from TPNH oxidation			
	pH 4.6	pH 2.3	pH 1.1	
			½ min[a]	30 min[a]
mM	*% of TPNH*			
0.0		37.0		
0.7	6.6	2.4	12.5	14
3.6	2.9	0.3	2.6	4

[a] These refer to the time in NaOH before acidification.

before significant reaction could occur; yet TPN^+ was produced equivalent to half of the hemoglobin iron present. It is conceivable, therefore, that TPNH is rapidly absorbed to hemoglobin and that the oxidation step is consequently not bimolecular.

The *rate* of reaction has not been studied as a function of temperature or pH, although it is clear that the rate diminishes as the pH is increased and becomes negligible in neutral or alkaline solution (except at high temperature; see below). However, the cumulative *yield* of TPN^+ is only moderately influenced by raising the temperature from 0 to 60° or lowering the pH from 2.3 to 0.6 (Table II). Consequently, the reaction rate must increase fast enough with temperature and H^+ to compensate for the greatly increased rate of destruction of hemoglobin and TPNH. For example, at 60° in 0.3 N HCl, the half-time of TPNH is probably of the order of 0.03 sec (5).

Oxidation of TPNH by Other Heme Compounds—The capacity of hemoglobin to oxidize TPNH in acid is rather specific. When tested in 0.1 N HCl, insignificant oxidation was produced by horse heart cytochrome *c*, catalase, or cytochrome b_5 (courtesy of Dr. Philipp Strittmatter) at absolute concentrations up to 15 μM and mole ratios to TPNH approaching 1:1. Conversion of oxyhemoglobin to methemoglobin, carbon monoxyhemoglobin, or even to reduced hemoglobin eliminated its oxidation capacity.

The question of whether hemoglobin is the only substance in liver capable of oxidizing TPNH on acidification will be taken up below.

Protection of TPNH from Oxidation by Hemoglobin in Acid—A number of reducing agents were tested for their ability to permit acid destruction of TPNH without oxidation to TPN^+ by hemoglobin. Polakis and Bartley (11) found that cysteine, added to weak acid used in making extracts of yeast, gave lower TPN^+ values, presumably by preventing TPNH oxidation. Neubert *et al.* (7) reported that reduced glutathione is partially protective against oxidation of TPNH when liver mitochondria are acidified. Tests were made of four sulfhydryl compounds: cysteine, mercaptoethanol, dithiothreitol, and reduced glutathione. These were all almost completely ineffective whether added to the acid (up to 8 mM concentration) or to an alkaline mixture of hemoglobin and TPNH prior to acidification (final —SH concentration up to 1.5 mM).

However, another reducing agent, ascorbic acid, proved to be quite effective. When ascorbic acid was added to an alkaline mixture of hemoglobin and TPNH, TPN^+ formation was reduced to a negligible value upon acidification to pH 2.3 (Table VI). Protection was not quite so effective at pH 1.1 or 4.6, but was nevertheless nearly complete. To be fully effective the ascorbic acid concentration must be at least 2 or 3 mM at the acidification stage but it proved to be equally protective whether it was added to an alkaline or neutral hemoglobin-TPNH mixture before acidification, or if it was added to the acidifying solution. In spite of the known susceptibility of ascorbic acid to oxidation in alkali, it was found that it could be kept in 0.04 N NaOH at 0° for at least 30 min, together with hemoglobin and TPNH, without losing its protective capacity (Table VI). Hemoglobin itself is unfortunately sufficiently stable in cold NaOH so that it was not found possible to destroy it in alkali without also destroying TPN^+ (not shown).

Oxidation and Protection of DPNH—Although the studies described were made with TPNH, DPNH is also oxidized (to DPN^+) by hemoglobin in acid solution and likewise the oxidation can be prevented with ascorbic acid. The rates of reaction of DPNH and TPNH with hemoglobin appear to be about equal, as shown by the following experiment. A mixture of hemoglobin with both DPNH and TPNH was prepared in neutral solution with equal amounts of each nucleotide, but slightly less hemoglobin (on an equivalent basis) than either of the nucleotides. Aliquots were diluted in acid (pH 2.3) and heated at 60°. The yield of DPN^+ and TPN^+ was measured and found to be nearly the same for each nucleotide (33 and 34%, respectively). Since hemoglobin was limiting, if the rates of oxidation had not been nearly equal, either DPN^+ or TPN^+ would have been expected to preponderate.

Since DPNH levels in tissues are ordinarily much lower than those of DPN^+, the oxidation of some DPNH to DPN^+ is not of so much consequence as in the case of TPNH. Nevertheless, for precise results it is desirable to prevent this oxidation.

Stability of TPN^+ and DPN^+ in NaOH—The finding of a solution to the problem of acidifying TPNH in the presence of hemoglobin suggested the possibility of using a single alkaline

homogenate for measuring both TPN^+ (or DPN^+) and TPNH (or DPNH). Dr. Franz M. Matschinsky (12) had found that at 0°, in 0.02 N NaOH, DPN^+ and TPN^+ are sufficiently stable so that an alkaline homogenate can be used for measuring total DPN and total TPN. It was nevertheless felt desirable to establish more exactly the tolerance of TPN^+ and DPN^+ at various temperatures between 0° and room temperature (Table VII). The table records the half-times of destruction. To calculate the time for 1% destruction the times shown are divided by 70. Thus at 0° in 0.04 N NaOH it would require 7 or 8 min for 1% destruction of DPN^+ or TPN^+, or 40 min for a 5% loss. It will be noted that there is a very large temperature coefficient with an approximate doubling of the rate for each 5° increase. TPN^+ is on the average about 15% more stable than DPN^+ under these conditions. The data supplement an earlier extensive report on stability of DPN^+ and DPNH as a function of pH and temperature (5).

TABLE VII

Effect of temperature and NaOH concentration on stability of TPN^+ and DPN^+

DPN^+ and TPN^+ were added to four concentrations of NaOH at four temperatures, as shown. The respective initial nucleotide concentrations were 15 and 28 μM. At appropriate intervals duplicate aliquots were assayed fluorometrically with the lactic dehydrogenase reaction for DPN^+ and the glucose-6-P dehydrogenase reaction for TPN^+. The times for one-half of the nucleotide to be destroyed were calculated and are shown in minutes.

Temperature	Nucleotide	NaOH concentration			
		0.02 N	0.04 N	0.1 N	0.25 N
0°	DPN^+	745	495	297	138
	TPN^+	980	550	343	156
5	DPN^+	334	231	145	69
	TPN^+	407	267	171	90
10	DPN^+	163	114	76	42
	TPN^+	184	123	75	42
20	DPN^+	42	30	20	14
	TPN^+	52	35	22	13

Validation of Procedure—To test the over-all procedure, rat livers were prepared for analysis in various ways (Table VIII). One group was perfused prior to freezing to test the influence of intrinsic blood on the results. It will be seen that without perfusion approximately half of the total TPN was found as TPN^+ in homogenates prepared in weak acid. In perfused livers the TPN^+ proportion was only one-sixth. Almost the same results were found in each case if the tissue was homogenized in NaOH before making it acid (no ascorbate). If, however, ascorbic acid was added to the NaOH homogenates of control livers before acidification, the TPN^+ found was sharply reduced (to one-eighth of the total). Even in the case of the perfused livers a reduction in TPN^+ was observed when ascorbate was added. (The lower absolute levels in the perfused livers are probably attributable to swelling.) The validity of the values obtained with ascorbic acid is confirmed by the agreement between the two estimates of total TPN, one based on the sum of TPN^+ and TPNH measured separately, and the other based on direct

TABLE VIII

Effect of method of sample preparation on apparent levels of TPN^+ and TPNH

Livers from adult male rats (205 to 250 g) anesthetized with ether were frozen either at once (Control) or after perfusion *in situ* through the hepatic vein for 2 min with 30 to 40 ml of cold (0°) isotonic saline (Perfused). From each liver, homogenates were made at the temperatures shown in either 200 volumes of 0.02 N H_2SO_4-0.1 M Na_2SO_4, (acid-sulfate), 100 volumes of 0.04 N NaOH, 400 volumes of Tris-HCl buffer, pH 8, or 100 volumes of CH_3OH. For TPN^+, the homogenates made in acid-sulfate were heated 30 min at 60°; the other homogenates were diluted 5- or 10-fold with acid-sulfate and then heated. For TPN^+ analyses under the heading "Ascorbate" the NaOH homogenates were made 30 mM in ascorbic acid before dilution with the acid. For TPNH, the homogenates were diluted 5- to 10-fold with 0.04 N NaOH containing 0.5 mM cysteine (freshly added) and heated 10 min at 60°. For total TPN (Direct) the unheated alkaline homogenates were analyzed directly after suitable dilution. In the case of the CH_3OH homogenate this dilution was made with CH_3OH. All analyses were made by enzymatic cycling. Concentrations are expressed as micromoles per kg, wet weight. The standard errors are shown in *italics* for four perfused livers and seven control livers.

Homogenate	TPN^+				TPNH		Total TPN			
	No ascorbate		Ascorbate				Sum		Direct	
	Control	Perfused	Control	Perfused	Control	Perfused	Control	Perfused	Control	Perfused
Acid-sulfate, 0°	274	76					760[a]	508[a]		
	22	*17*								
NaOH, 0°	256	65	63	42	486	432	549[b]	474[b]	537	456
	8	*8*	*3*	*3*	*7*	*23*			*10*	*13*
Tris, pH 8, 80°	149	42			390	457	539	499	526	475
	22	*28*			*12*	*16*			*14*	*24*
CH_3OH, −20°					473				540	
					9				*1*	

[a] Sum of TPN^+ plus TPNH measured in the NaOH homogenate.

[b] Sum of TPNH plus TPN^+ from the ascorbate column.

TABLE IX

Recovery of TPN+ and TPNH added to liver and blood

TPN+ and TPNH were added as a mixture to homogenates of liver in 0.04 N NaOH containing 0.5 mM cysteine, and to blood diluted in the same medium. The analyses were conducted by the regular procedures described. The additions and results are recorded as micromoles per kg of liver or per liter of blood.

Tissue	Nucleotide	No addition	Amount added	Total	
				Calculated	Found
Liver A	TPN+	74	56	130	130
Liver B	TPN+	50	56	106	106
Liver A	TPNH	433	110	543	543
Liver B	TPNH	465	110	575	580
Blood A	TPN+	5.6	6.4	12.0	12.1
Blood B	TPN+	3.3	10.1	13.4	14.1
Blood C	TPN+	7.9	10.7	18.6	18.9
Blood A	TPNH	16.1	10.3	26.4	25.8
Blood B	TPNH	6.1	17.9	24.0	24.7
Blood C	TPNH	15.9	14.0	29.9	30.2
Blood A	Total TPN[a]	22.3	16.7	39.0	39.4
Blood A	Sum[b]	21.7	16.7	38.4	37.9
Blood C	Total TPN[a]	25.0	24.7	49.7	50.1
Blood C	Sum[b]	23.8	24.7	48.5	49.1

[a] TPN+ plus TPNH measured together in the cold NaOH dilution of blood.

[b] Sum of TPN+ plus TPNH each determined separately above. The measurements of TPN+ and TPNH separately and together were made on the same blood sample in each case.

TABLE X

TPN+ and TPNH contents of rat kidney, heart, brain, and blood

Male rats (240 to 280 g) were lightly anesthetized with ether for 1 min and the organs (or whole head in the case of the brain) were frozen in Freon-12 at −150° within 5 sec of the time of cutting off the blood supply. The renal vessels were clamped as the kidney was removed, after which the heart was also rapidly taken. A separate set of animals were used for brain. The analyses were made by the regular procedure described except that the whole organs were powdered at −180°, and sampled by weighing at −20°. Values are recorded as micromoles per kg or liter and represent averages of the number of animals shown in parentheses. The standard errors are also shown.

Tissue	TPN+	TPNH	Total TPN	
			Sum	Direct analysis
Kidney (5)	19 ± 1	135 ± 7	154	148 ± 7
Heart (5)	4.5 ± 0.4	93 ± 5	98	95 ± 5
Brain (4)	5.3 ± 0.3	26.4 ± 1	31.7	25 ± 1
Blood (9)	5.4 ± 0.2	15.2 ± 0.5	20.6	19.2 ± 0.3

analysis of the two forms together in the alkaline homogenates. From these results it appears that blood is responsible for most, but perhaps not quite all, of the TPNH oxidation in acid, and that ascorbic acid can protect against both intrinsic and added blood.

Homogenates were also prepared by heating at 80° in Tris buffer at pH 8. It will be seen (Table VIII) that with control livers this resulted in substantially lower values for TPNH with corresponding increases in TPN+. This oxidation can again be attributed to blood, since an increase in TPN+ was not observed if the livers had been perfused before heating in Tris buffer. When frozen tissue is homogenized at 0°, there is in principle a brief period of thawing before the homogenizing fluid makes intimate contact with all tissue elements. To see whether there might be any oxidation or reduction of TPN during this homogenization, samples of the two livers were homogenized at −10° in NaOH containing 2 M KCl to lower the freezing point. In this case no thawing of the tissue can occur until after it is made alkaline. Values for TPN+ and TPNH were obtained which were identical with those found in homogenates made in alkali at 0° (not shown). Similarly, the liver homogenates prepared without thawing in CH_3OH at −20° had the same TPNH and total TPN content as those prepared in NaOH at 0° (Table VIII).

A final validation of the over-all procedure, and confirmation of the protective effect of ascorbic acid, are afforded by recovery experiments with TPN+ and TPNH added to alkaline homogenates of normal liver and to blood itself (Table IX). There is no sign of any conversion of TPNH to TPN+ in either the alkaline homogenate or upon acidification to destroy TPNH. Nor is there any sign of TPN+ destruction in the cold alkali.

Normal Values for TPN+ and TPNH—In addition to liver, shown above, kidney, heart, brain, and blood were analyzed by the proposed procedures (Table X). The proportion of TPN+ varied from a high of 26% in blood to a low of 4.5% in heart. Total TPN measured directly agreed to within 4% with the sum of the separate analyses in the case of kidney and heart, and to within 7% in the case of blood. In brain, however, the value by direct analysis was 20% lower than the sum. Although this could mean that some TPNH was oxidized at the time of acidification, it is believed more likely that this results from some destruction of TPN by DPNase (also active toward TPN+) during the cycling step. Tissue DPNase is not completely destroyed by the cold alkali, and in the case of brain, because of low TPN content and exceptionally active DPNase, it is not easy to dilute sufficiently to prevent some enzyme action. A need for further study of conditions for total TPN measurement in brain is indicated.

Comparison of TPN+ and TPNH Values Reported Here with Those Obtained by Others—In the case of the liver comparisons are made somewhat uncertain by the fact that total TPN values are higher in male rats than in females, and that some reports are for one sex and some for the other. Nevertheless it seems fair to conclude that the proposed procedure gives TPNH:TPN+ ratios and values for the sum of TPN+ plus TPNH that are in the upper range of those in the literature (Table XI).

There are fewer opportunities for comparison with the literature in the case of other tissues. Klingenberg (14) reported values for TPNH in heart and brain that are very similar to those reported here (91 and 27 μmoles per kg, respectively), but levels for TPN+ much higher than those given here (10-fold higher in heart and 3-fold higher in brain). Glock and McLean (21) on the other hand found about the same amount of TPN+ in heart as reported here but the average for TPNH was only one-third as great. In brain Glock and McLean found both TPN+ and TPNH levels that are only one-third or less of those

TABLE XI

Comparison of TPN+ and TPNH values in rat liver reported by various authors

Sex	TPN+	TPNH	Total	Ratio of TPNH to TPN	Authors
	μmoles/kg				
	30	250	280	8.3	Bassham *et al.* (13)
	113	315	418	2.8	Klingenberg (14)
M	115	502	617	4.3	Lowry *et al.* (9)
M	141	447	588	3.2	Christie and Le Page (15)
F	34	238	273	7.2	Slater *et al.* (16)
M	59	355	415	5.9	Slater *et al.* (16)
	70	220	290	3.2	Bücher *et al.* (17)
F	70	310	380	4.4	Chance *et al.* (18)
F	65	311	376	4.8	Heldt *et al.* (4)
F	59	360	419	6.1	Neuhoff and Desselberger (19)
M	31	278	309	9.0	Clark *et al.* (20)[a]
M	63	486	549	7.7	This paper

[a] The values given here represent the mean of three different control groups in which TPNH:TPN+ ratios varied from 7.2 to 14.2.

those given in Table X. Neuhoff and Desselberger (19) report values in kidney of 27 and 108 μmoles per kg, respectively, for TPN+ and TPNH, *i.e.* values that are somewhat higher for TPN+ and somewhat lower for TPNH than reported here. In regard to blood the present procedure gives about the same value as that reported by Glock and McLean (21) for TPN+ but a 5-fold higher value for TPNH.

DISCUSSION

From the data presented it seems clear that when tissues are homogenized in acid there is great danger of oxidation of some of the TPNH present to TPN+. This danger increases if any red cells present have been ruptured by freezing. Free hemoglobin is capable of oxidizing TPNH as effectively in strong acid as in weak. Nevertheless, a greater degree of TPNH oxidation occurs when frozen tissue is homogenized in dilute than in strong acid. The explanation must be that in dilute acid the destruction of hemoglobin is delayed long enough to permit mixing with TPNH, whereas a strong acid, particularly if it is a protein precipitant, destroys or precipitates hemoglobin before mixing is complete. This is borne out by the fact that more TPN+ is found in strong $HClO_4$ homogenates of frozen liver homogenized at 0° than in those homogenized at −10° (6). Still greater values are obtained if the tissue is allowed to thaw briefly before homogenization in strong $HClO_4$ (6).

One question is whether hemoglobin is solely responsible for the conversion of TPNH to TPN+ in acid. It was seen that although perfusion lowered apparent TPN+ values in liver, ascorbate was capable of reducing the TPN+ level still further. Since failure to remove as little as 0.2% of blood from the liver would explain this result, the experiment does not settle the question. The same can perhaps be said for the finding of Neubert *et al.* (7) and Heldt *et al.* (8) that when mitochondria are treated with dilute acid, oxidation of TPNH and DPNH to TPN+ and DPN+ can occur. The presence of an almost undetectably small number of red blood cells in the mitochondrial preparations could explain the phenomenon. That adventitious materials, possibly red cells, may be responsible for these findings is suggested by the fact that with liver mitochondria, kindly prepared for us by Dr. F. E. Hunter in various metabolic states, there was little or no difference in TPN+ values whether the mitochondrial samples were homogenized in dilute acid (pH 2.3) or in 0.3 N $HClO_4$. In any event, hemoglobin appears to be the worst offender in causing erroneously high TPN+ values in animal tissues.

The proposed procedure circumvents the hemoglobin problem by making the original extract in alkali and then dividing the sample for separate measurement of TPN+ and TPNH. This has the advantage that both forms of the nucleotide are measured in the same sample; in fact the same sample could be used for DPN+ and DPNH analyses as well.[3]

Heldt *et al.* (4) have devised a method for measuring the pyridine nucleotides in a single acid extract. The sample is homogenized with $HClO_4$. The subsequent extract contains TPN+ and DPN+ plus the acid destruction products of the reduced pyridine nucleotides, adenosine diphosphoribose and P-adenosine diphosphoribose. These are separated chromatographically. It is evident from the foregoing that this method would also give erroneously high results for TPN+ or DPN+ if any free hemoglobin were present at the time of acidification.

The oxidation of TPNH by hemoglobin upon acidification, and its prevention by ascorbic acid, is not entirely unexpected. It has long been known that a number of compounds including ascorbic acid are oxidized by oxyhemoglobin when acidified (22). Moreover, the capacity to oxidize ascorbic acid *rapidly* is lost within a few seconds, as noted above for TPNH oxidation. Also, as shown here for TPNH, the capacity to oxidize ascorbic acid is lost if the oxyhemoglobin is reduced or converted to carbon monoxyhemoglobin before acidification. It appears likely that during hemoglobin destruction in acid an unstable intermediate, capable of oxidizing TPNH, is formed. If so, then ascorbic acid, when present at high enough concentration, reacts faster than TPNH with the intermediate.

REFERENCES

1. GREENGARD, P., BRINK, F., JR., AND COLOWICK, S. P., *J. Cell. Comp. Physiol.*, **44**, 395 (1954).
2. SPIRTES, M. A., AND EICHEL, H. J., *Arch. Biochem.*, **53**, 308 (1954).
3. NEUHOFF, V., *Arch. Exp. Pathol. Pharmakol.*, **250**, 302 (1965).
4. HELDT, H. W., KLINGENBERG, M., AND PAPENBERG, K., *Biochem. Z.*, **342**, 508 (1965).
5. LOWRY, O. H., PASSONNEAU, J. V., AND ROCK, M. K., *J. Biol. Chem.*, **236**, 2756 (1961).
6. BURCH, H. B., LOWRY, O. H., AND VON DIPPE, P., *J. Biol. Chem.*, **238**, 2838 (1963).
7. NEUBERT, D., SCHULZ, H-U., AND HOEHNE, R., *Biochim. Biophys. Acta*, **92**, 610 (1964).
8. HELDT, H. W., GREIF, N., KLINGENBERG, M., SCHOLZ, R., PANTEN, U., GRUNST, J., AND BÜCHER, T., *J. Biol. Chem.*, **240**, 4659 (1965).
9. LOWRY, O. H., PASSONNEAU, J. V., SCHULZ, D. W., AND ROCK, M. K., *J. Biol. Chem.*, **236**, 2746 (1961).
10. LOWRY, O. H., PASSONNEAU, J. V., HASSELBERGER, F. X., AND SCHULZ, D. W., *J. Biol. Chem.*, **239**, 18 (1964).
11. POLAKIS, E. S., AND BARTLEY, W., *Biochem. J.*, **99**, 521 (1966).
12. MATSCHINSKY, FRANZ M., *Fed. Proc.*, **25**, 753 (1966).
13. BASSHAM, J. A., BIRT, L. M., HEMS, R., AND LOENIG, U. E., *Biochem. J.*, **73**, 491 (1959).
14. KLINGENBERG, M., *Zur Bedentung der Freien Nucleotide 11.*

[3] If it is nevertheless desirable to use a separate sample for TPN+ or DPN+ analysis, it has been found satisfactory to prepare homogenates in the H_2SO_4-sulfate mixture containing 3 mM ascorbic acid. As suggested, it is also possible to make suitable homogenates in 2 M $HClO_4$ at −10° (6) or in an ethanol-$HClO_4$ mixture at −30° (8).

Colloquium der Gesellshaft für Physiologische Chemie, April 1960, Mosbach, Baden, Springer-Verlag, Berlin, 1961, p. 82.
15. Christie, G. S., and Le Page, R. N., *Biochem. J.*, **84,** 25 (1962).
16. Slater, T. F., Sawyer, B., and Sträuli, U., *Arch. Int. Physiol. Biochim.*, **72,** 427 (1964).
17. Bücher, T., Krejci, K., Russmann, W., Schnitger, H., and Wesemann, W., in B. Chance, R. H. Eisenhardt, Q. H. Gibson, and K. K. Lonberg-Holm (Editors), *Symposium on Rapid Mixing and Sampling Techniques in Biochemistry,* Academic Press, New York, 1964, p. 255.
18. Chance, B., Schoener, B., Krejci, K., Russmann, W., Wesemann, W., Schnitger, H., and Bücher, T., *Biochem. Z.*, **341,** 325 (1965).
19. Neuhoff, V., and Desselberger, E., *Arch. Exp. Pathol. Pharmakol.*, **252,** 43 (1965).
20. Clark, J. B., Greenbaum, A. L., and McLean, P., *Biochem. J.*, **98,** 546 (1966).
21. Glock, G. E., and McLean, P., *Biochem. J.*, **61,** 388 (1955).
22. Lemberg, R., and Legge, J. W., *Hematin compounds and bile pigments,* Interscience Publishers, Inc., New York, 1949, pp. 402, 403.